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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 5 : C12Q 1/68, C07H 15/12, C12N 15/00 — **A1**

(11) International Publication Number: WO 91/11535

(43) International Publication Date: 8 August 1991 (08.08.91)

(21) International Application Number: PCT/US91/00635

(22) International Filing Date: 30 January 1991 (30.01.91)

(30) Priority data:
472,137 30 January 1990 (30.01.90) US
Not furnished 29 January 1991 (29.01.91) US

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(81) Designated States: AT (European patent), AU, BB, BE (European patent), BF (OAPI patent), BG, BJ (OAPI patent), BR, CA, CF (OAPI patent), CG (OAPI patent), CH (European patent), CM (OAPI patent), DE (European patent), DK (European patent), ES (European patent), FI, FR (European patent), GA (OAPI patent), GB (European patent), GR (European patent), HU, IT (European patent), JP, KR, LK, LU (European patent), MC, MG, ML (OAPI patent), MR (OAPI patent), MW, NL (European patent), NO, PL, RO, SD, SE (European patent), SN (OAPI patent), SU, TD (OAPI patent), TG (OAPI patent).

Published
With international search report.

(54) Title: INHIBITION OF TRANSCRIPTION BY DOUBLE-STRANDED OLIGONUCLEOTIDES

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                    E1b -65 TO +50

   -60      -50      -40      -30      -20      -10
    .        .        .        .        .        .
TGCATGGCGTGTTAAATGGGGCGGGGCTTAAAGGGTATATAATGCGCCGTGGGCTAATCTTGGTT

            GATCGGGGCGGGGC     14-mer
               CCCCGCCCCGCTAG

      ACGTTGCAGCCGGGGCGGGGCTTCTGCA     28-mer
          ACGTCGGCCCCGCCCCGAAGACGTTGCA

1        10       20       30       40       50
.        .        .        .        .        .
ACATCTGACCTCATGGAGGCTTGGGAGTGTTTGGAAGATTTTTCTGCTGTGC
                              CCTTCTAAAAAGACGACACG
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(57) Abstract

The present invention relates to novel methods for controlling gene expression in which double stranded oligonucleotides are used to inhibit the interaction of transcriptional factors with transcriptional control elements in DNA. The methods of the invention are particularly useful in selectively inhibiting transcription of viral genes and oncogenes, and may be used in the treatment of a variety of viral diseases. In preferred embodiments of the invention, nucleosides are joined by phosphorothioate linkage.

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INHIBITION OF TRANSCRIPTION BY DOUBLE-STRANDED OLIGONUCLEOTIDES

1. INTRODUCTION

The present invention relates to novel methods for controlling gene expression in which double stranded oligonucleotides are used to inhibit the interaction of transcriptional factors with transcriptional control elements in DNA. The methods of the invention are particularly useful in selectively inhibiting transcription of viral genes and oncogenes, and may be used in the treatment of a variety of viral diseases. In preferred embodiments of the invention, nucleosides are joined by phosphorothioate linkage.

2. BACKGROUND OF THE INVENTION

The rate of initiation of transcription is regulated by cis-acting promoter and enhancer elements. The mechanism for this control involves binding of DNA sequence-specific proteins to these elements (Gidoni et al., 1984, Nature, 312:409-413; Scholer, H. R. and Gruss, P., 1984, Cell, 86:403-411; and McKnight, S. L. and Kingsburg, R., 1982, Science, 217:316-324). Regulatory elements and binding proteins have been identified by in vivo functional studies with plasmids or viruses containing mutated regions or competing binding sites. Alternatively, in vitro binding assays, such as footprinting and gel retardation analysis, have been useful in delineating promoter function (Wu, C., 1986, Nature, 1985, 317:84-87 and Singh et al., 1986, Nature, 319:154-158). These studies have characterized both ubiquitous factors that bind to diverse regulatory elements and are present in nuclear extracts from many different cell types and unique factors that bind to few promoters or enhancers and are

present in only a specialized cell type or stage of differentiation (Rosales et al., 1987, EMBO J., 6:3015-3025; Peterson et al., 1988, Mol. Cell. Biol. 6:4168-4178).

2.1. TRANSCRIPTION FACTORS AND THEIR ROLES IN GENE EXPRESSION

A wide variety of transcription factors have been described. Transcription factors (TFs) have been identified which are the products of oncogenes, such as the fos protein (Lucibello et al., 1988, Oncogene 3:43-52) and v-jun protein (Bos et al., 1988, Cell 52:705-712). A TF has been found to be associated with the epidermal growth factor (EGF) receptor gene (Kageyama et al., 1988, Proc. Natl. Acad. Sci. U.S.A. 85:5016-5020). Other TFs have been observed to function in a tissue specific manner, such as lymphoid-specific TFs (Muller et al., 1988, Nature 336:544-551; Scheidereit et al., 1988, Nature 336:551-557), the liver specific TF LF-B1 (Frain et al., 1989, Cell 59:145-157) and pituitary-specific TFs (Bodner et al., 1988, Cell 55:505-518; Ingraham et al., 1988, Cell 55:519-529). A convulsant-induced increase of TF-encoding mRNA has been observed in rat brain (Saffen et al., 1988, Proc. Natl. Acad. Sci. U.S.A. 85:7795-7799) and Morgan et al. (1987, Science 237:192-197) reported that c-fos mRNA was induced in rat brain by seizures.

A number of viral TF or cellular TFs which interact with viral promoter/enhancer elements have been identified. These include TF derived from SV40, which appears to activate the SV40 late promoter in vitro (Beard and Bruggmann, 1988, J. Virol. 62:4296-4302), and nuclear factor EF-C, which occurs in human HepG2 liver cells and in other nonliver cell lines and which is observed to bind to the hepatitus B virus and polyoma virus transcriptional enhancer regions in vitro (Ostapchuk et al., 1989, Mol. Cell. Biol. 9:2787-2797). Another interesting TF is the

papillomavirus E2 transactivator protein. The E2 open reading frame of bovine papillomavirus type 1 encodes at least three TFs. These include positive as well as negative regulators of transcription (Lambert et al., 1987, cell 50:64-78; McBride et al., 1988 EMBO J. 7:533-540; Lambert et al., 1989, J. virol 63:3151-3154).

Human immunodeficiency virus type 1 (HIV-1), which causes acquired immunodeficiency syndrome (AIDS), has been found to utilize a number of different TFs in its mode of gene expression, which interact with both viral and cellular proteins. Five regions of the HIV-1 long terminal repeat (LTR) region, including the negative regulatory enhancer, SP1, TATA, and TAR regions, have been shown to be important in the transcriptional regulation of HIV genes (Garcia et al., 1989, EMBO J. 8:765-778; Harrich et al., 1989, J. Virol. 63:2585-2591). The enhancer element has been found to contain two copies of the sequence GGGACTTCC (which shares homology with the eukaryotic TF NF-kappa B); the TAR region, located between -17 and +44 in the viral genome, contains two copies of the sequence CTCTCTGG (Wu et al., 1988, EMBO J. 7:2117-2130). Cellular protein EBP-1 has been observed to bind to the enhancer region, whereas cellular protein UBP-1 appears to bind to the TAR region (Wu et al., ibid). Further, a protein encoded by the human T cell leukemia virus I (HTLV-I) tax gene has been found to interact with the HIV-1 enhancer region. The HIV-1 transcriptional transactivator protein tat has also been shown to interact with the TAR region (Arya et al., 1985, Science 229: 69-73; Fisher et al., 1986, Nature 320:367-371; Kao et al., 1987, Nature 330:489-493).

Three SP-1 binding sites in the HIV LTR were found to be important to in vitro transcription from the HIV-LTR promoter. Harrick et al. (1989, J. Virol. 63:2585-2591) observed that mutagenesis of the HIV-1 LTR

SP-1 sites, which converted them to consensus high affinity SP-1 binding sites, resulted in an increase of tat-induced gene expression.

SP-1 is a ubiquitous factor which increases transcription of RNA polymerase II 10 to 50 fold from promoters that contain one or more hexanucleotide sequences, GGGCGG, called GC boxes (Gidoni, supra and Briggs et al., 1986, Science, 234:47-52).

The E1B transcriptional unit promoter has only a single SP1 binding site and a TATA box (Wu et al., 1987, Nature, 326:512-515). Deletion and linker scan substitution of the SP1 site in the adenovirus E1B promoter produced mutant viruses that yielded only 13 to 20% of the basal transcription of wild type virus after infection of HeLa cells (Wu, supra).

2.2. OLIGONUCLEOTIDE INHIBITORS OF TRANSCRIPTION

Currently, investigators are attempting to control the expression of genes with single stranded antisense oligonucleotides. Antisense oligonucleotides bind to complementary mRNA sequences and block translation or cause cleavage of the double stranded duplexes formed (Stein, C. A. and Cohen, J. S., 1988, Cancer Res., 48:2659-2668). This approach can decrease expression of specific genes but cannot be generally applied because of limited binding of the oligonucleotide to mRNA regions of strong secondary structure, poor transport into cells and rapid degradation. More stable chemically modified oligonucleotide have multiple stereoisomers that may decrease binding to mRNA (Zon, 1988, supra).

Other investigators have explored the use of double-stranded oligonucleotides in controlling gene expression (European Patent Application No. 88307302.5; Androphy et al., 1987, Nature 325:70-72), but have faced

the problem that such oligonucleotides do not pass readily into cells, and are susceptible to degradation. For example, Hawley-Nelson et al. (1988, EMBO 7:525-531) inserted oligonucleotides into a plasmid and then used standard calcium phosphate transfection techniques to introduce the oligonucleotides into eukaryotic cells. Such transfection techniques, to date, cannot be feasibly applied to introduce DNA into living organisms.

3. SUMMARY OF THE INVENTION

The present invention relates to novel methods for controlling gene expression in which double stranded oligonucleotides are used to compete for the binding of nuclear factors to specific cellular transcriptional control elements. The invention is based in part on the discovery that oligonucleotides containing a GC box can specifically inhibit transcription of E1B.

In various embodiments of the invention, an oligonucleotide comprising one or, preferably, more than one binding site for a transcription factor may be used to inhibit the transcription of genes under the control of promoter/enhancer elements which bind to said transcription factor. In preferred embodiments of the invention, the transcription factor is a viral transcription factor, and the method of the invention may be used in the treatment of viral diseases, such as retroviral diseases, in humans or animals. In other preferred embodiments of the invention, the transcription factor binds to a control element of an oncogene or growth factor, and the method of the invention may be used in the treatment or prevention of cancer. According to the most preferred embodiments of the invention, the nucleosides of the oligonucleotide are joined by phosphorothioate linkage.

In an alternate embodiment of the invention, a oligonucleotide comprising one or more than one binding site for a transcription factor may be used to increase the transcription of genes normally repressed by said transcription factor.

According to a specific embodiment of the invention, oligonucleotides containing a SP1 binding sequence may be used to inhibit transcription of E1B. In a preferred specific embodiment of the invention, oligonucleotides may comprises multiple copies of SP1 binding sequences.

4. DESCRIPTION OF THE FIGURES

Figure 1. Nucleotide sequence of the E1B transcriptional unit and the inhibitor oligonucleotides.

The first line is the promoter region of E1B with the GC and TATA boxes underlined. The sequences of the annealed 14 mers and 28 mers are shown on the next 4 lines oriented below the homologous GC box in the E1B promoter. The transcribed portion of E1B (+1 to +52) is shown on the next line with the synthetic oligonucleotide used for the primer extension oriented below.

Figure 2. Radioautograph of a transcription assay. The heavy bands at the bottom are the 20 base oligomer used for the primer extension. The 50 base bands are the expected transcript for the E1B unit shown in Fig. 1. Reactions in lane 1 and 2 contained no competing oligonucleotides. Lanes 3 and 4 contained 0.22 and 0.87 μg 14 mer, SP1S. Lanes 5 and 6 contained 0.2 and 1.0 μg of the 28 mer, 17/19; lanes 7, 8 and 9 contained 0.12, 0.8 and 1.0 μg of 21/25; and lanes 10, 11 and 12 contained 0.1, 0.8 and 1.0 μg of 18/20. M is a standard lane of molecular weight markers containing labeled <u>Hpa</u>II digest of pBR322.

Figure 3. Inhibition of transcription by varying concentrations of oligonucleotides with one, two or three SP1 sites. The lines are best fit regressions through the data.

Figure 4. Uptake of radiolabelled phosphodiester-linked (open circles) and phosphorothioate linked (closed circles) oligonucleotides by MOLT 4 cells.

5. DETAILED DESCRIPTION OF THE INVENTION

The present invention relates to novel methods for controlling gene expression in which double stranded oligonucleotides are used to competitively inhibit the binding of transcription factors to specific transcriptional control elements in DNA. For purposes of clarity of disclosure, and not by way of limitation, the detailed description of the invention is divided into the following subsections:

- (i) transcription factors which may be inhibited according to the invention;
- (ii) identification of oligonucleotides that may be used to inhibit transcriptional factor binding to control elements;
- (iii) oligonucleotides of the invention; and
- (iv) utility of the invention.

5.1. TRANSCRIPTION FACTORS WHICH MAY BE INHIBITED ACCORDING TO THE INVENTION

According to the invention, double stranded DNA oligonucleotides may be used to inhibit any transcriptional factor which influences transcription by binding to controlling elements of a gene. In particular embodiments of the invention, the transcriptional factor acts to increase transcription of a gene, for example, by binding

to a promoter/enhancer element. In alternate embodiments of the invention, the transcriptional factor acts to repress transcription of a gene. Examples of transcriptional repressors include, but are not limited to, the bovine papillomavirus E2 gene. Examples of transcription factors which increase transcription include, but are not limited to, those listed in Table I.

TABLE I

Transcription Factor	Reference
lymphoid-specific	Muller et al., 1988, Nature 336:544-551 Scheidereit et al., 1988, Nature 336:551-557
LF-B1 (liver-specific)	Frain et al., 1989, Cell 59:145-157
Pituitary Specific	Bodner et al., 1988, Cell 55:505-518 Ingraham et al., 1988, Cell 55:519-529
Active on EGF Receptor Gene	Kageyama et al., 1988, Proc. Natl Acad. Sci. USA 85:5016-5020
Fos protein	Lucibello et al., 1988, Oncogene 3:43-52
v-*jun* protein	Bos et al., 1988, Cell 52:705-712
PEA 1	Wasylyk et al., 1988, EMBO J. 1:2475-2483 [&]
EF-C	Ostapchuk et al., 1989, Mol. Cell. Biol. 9:2787-2797

According to preferred embodiments of the invention, oligonucleotides may be used to inhibit the function of transcription factors which are capable of increasing transcription by competing with native DNA binding sites for transcription factor, thereby effectively inhibiting the transcription of a gene (or genes) which is (are) influenced by said transcription factor. For example, and not by way of limitation, double stranded DNA oligonucleotides which bind to a transcription factor which induces the transcription of a viral gene may compete with viral promoter/enhancer elements for transcription factor binding and thereby effectively inhibit the transcription of viral gene sequences.

In alternate embodiments of the invention, double stranded DNA oligonucleotides may be used to inhibit the function of transcription factors which are capable of repressing transcription by competing with native DNA binding sites for the repressor, thereby effectively increasing the transcription of a gene (or genes) which is (are) influenced by said transcription factor. For example, and not by way of limitation, double stranded DNA oligonucleotides which bind to a viral repressor protein which normally renders a viral gene or genes functionally inactive may be used to activate the expression of these viral genes in a controlled manner; this method may prove useful in the study of latent viral infection in animal models.

Importantly, a particular transcription factor need not be characterized in order to be inhibited according to the present invention. It would be sufficient for a DNA sequence which influences transcription to be identified. For example, the transcription of a particular gene under study may be found to be controlled by a mechanism which includes the presence of a particular DNA sequence; such a sequence might be identified by studying

mutations of the gene and its surrounding DNA sequences. Mutation of a sequence important in promoting transcription (a promoter element) may be found to result in a relative decrease in the transcription of a particular gene (either the gene naturally associated with the promoter element or a reporter gene put under the control of the promoter element). Also, DNA sequences which bind to potential transcription factors may be identified by footprinting techniques or gel retardation analysis using standard techniques known in the art. The DNA sequence of the binding site may then be determined using standard sequencing techniques (for example, Sanger et al., 1979, Proc. Natl. Acad. Sci. U.S.A. 72:3918-3921).

DNA sequences which bind to repressor transcription factors may be identified in an analogous manner.

5.2. IDENTIFICATION OF OLIGONUCLEOTIDES THAT MAY BE USED TO INHIBIT TRANSCRIPTION FACTOR BINDING TO CONTROL ELEMENTS

Oligonucleotides which may be used to inhibit transcription factor binding to control elements may be identified by determining whether said oligonucleotides (a) bind to said transcription factor and/or (b) inhibit the function of said transcription factor.

Oligonucleotides may be tested for an ability to bind to a transcription factor by any method known in the art, including, but not limited to, the following.

If a transcription factor (TF) has been characterized and purified, the capability of an oligonucleotide may be tested directly for binding to the TF. For example, TF may be immobilized, and then exposed to labeled oligonucleotide, upon which selective retention of labeled oligonucleotide to TF could be measured.

Whether a TF has or has not been characterized, but it has been shown, by DNase footprinting analysis (Galas and Schmitz, 1978, Nucl. Acids Res. 5:3157-3170; Bos et al., 1988, cell 52:705-712), to bind to a particular DNA sequence, the binding capability of an oligonucleotide may be tested indirectly. For example, oligonucleotide may be included in the reaction in which TF (in purified or unpurified form) is allowed to bind to DNA; the oligonucleotide may be observed to competitively inhibit the binding of TF to its target sequence, thereby diminishing the appearance of a clear "footprint."

Alternatively, characterized or uncharacterized, purified or unpurified TF may be tested for the ability to bind an oligonucleotide using gel retardation analysis (Barberis et al., 1987, cell 50:347-359). For example, an oligonucleotide could be exposed to purified TF or, alternatively, TF as found in a mixture (e.g. a nuclear extract) under conditions which may allow binding of TF to the oligonucleotide. When subjected to polyacrylamide gel electrophoresis, the mobility of oligonucleotide bound to TF would be expected to be retarded relative to the mobility of unbound oligonucleotide.

Alternatively, oligonucleotides may be tested for the ability to inhibit the function (e.g. as inducer or repressor) of said transcription factor. Oligonucleotides may be evaluated using transcription systems in vitro or in vivo which comprise a control element which is believed to interact with a transcription factor and which controls the expression of a test gene.

For example, and not by way of limitation, in a specific embodiment of the invention which is exemplified in Section 6, infra, the ability of an oligonucleotide to inhibit transcription of the adenovirus E1B gene may be tested in vitro as follows. Nuclear extracts may be prepared from actively growing cells as described in

Section 6.1.3., infra. Transcription mixtures may contain the following: about 0.5-1.2 μg of DNA comprising a test gene under the control of an element which is believed to bind to a transcription factor in a final concentration of 226 mM HEPES pH 7.9, 48 mM KCl, 6 mM $MgCl_2$, 9.6% glycerol, 0.1 mM EDTA, 0.6 mM of ATP, GTP, CTP and UTP, 0.5 mM dithiothreitol and 0.5 mM phenylmethylsulfonylfluoride, and competitor oligonucleotide (0.1 to 1.0 μg/reaction; preferably, a range of amounts are tested). Reaction may be started by addition of extract (generally about 1-2 μg protein/μl nuclear extract) incubated 30-90 minutes at 30°C, and terminated by addition of a mixture of 175 μg of 200 mM NaCl,20 mM EDTA and 1% sodium dodecyl sulfate, 20 μg purified yeast tRNA, followed by extraction with 100 μg phenol and 100 μg chloroform-isoamyl alcohol (19:1) to each tube. The oligonucleotides in the aqueous phase may be precipitated with 0.5 M NH_4 acetate and 3 volumes of ethanol, resuspended in 200 μg 0.3 M Na acetate pH6, reprecipitated with 3 volumes of ethanol and dried in a vacuum centrifuge.

The mRNA products of transcription in this, or any transcription assay, may be analyzed by any method known in the art, including, but not limited to, Northern blot analysis and/or quantitative hybridization (e.g. hybridization of labeled mRNA to DNA immobilized on filters). In a preferred embodiment of the invention, a primer extension assay, such as that developed with E1B for analysis of transcriptional factors (see Section 6.1.2. and 6.2.1., infra) may be used; in particular, the residue from the aqueous phase of the transcription reaction may be dissolved in 10 μg of 0.25 M KCl in TE buffer containing 0.17-0.24 ng of oligonucleotide primer 5'-phosphorylated with [^{32}P]-ATP and annealed at 65° for about 30 minutes. The solution may then be cooled and the primer extended by incubation for about 30 minutes at 37°C in a solution

containing 14 mM tris buffer pH8, 7 mM $MgCl_2$, 3.5 mM DTT, 0.2 mM each of dATP, dGTP, dCTP and DTTP, 7 μg/ml of actinomycin D and 0.085 U/μl Maloney murine leukemia virus reverse transcriptase in a final volume of about 35 μl. Following the reaction, the nucleic acid may be precipitated with 0.3 M Na acetate and 3 volumes of ethanol, dried, and dissolved in 10 μg buffered formamide containing bromphenol blue and xylene cyanol, then subjected to polyacrylamide gel electrophoresis at 300-400V in a 8 M urea, 10% acrylamide, 0.3% bis-acrylamide gel. Bands may be cut from dried gels and quantified by liquid scintillation counting.

As another example, the activity of a TF may be evaluated in vitro in a system which comprises the TF and a transcription template that includes a reporter gene under the control of a promoter which binds to said TF. The effect of test oligonucleotides on reporter gene expression may be expected to reflect the effectiveness of the oligonucleotide in inhibiting TF binding. For example, and not by way of limitation, an in vitro system may be utilized in which HIV tat protein is the TF and the transcription template is a reporter gene, such as the gene encoding chloramphenicol acetyltransferase (CAT), under the control of the HIV-1 LTR promoter. In such a system, double stranded oligonucleotides comprising one or more SP1 or NF kappa B sites, or both, may inhibit tat activity.

Alternatively, transcription may be carried out in isolated nuclei or whole cells which comprise the control element which is believed to bind to transcription factor, such that inhibition of TF binding to the control element and consequent inhibition of TF function is detectable. For example, the amount of RNA transcribed from a gene controlled by said control element may be measured in the presence or absence of oligonucleotide in pulse-labeling experiments of cells or isolated nuclei.

Alternatively, a protein product of a gene controlled by said control element may be monitored in the presence or absence of oligonucleotide; proteins suitable may include, but are not limited to, standard reporter genes such as chloramphenicol/acetyltransferase, β-galactosidase, luciferase, etc.

For example, a transcription template comprising a reporter gene and a control element may be transfected into cells which contain a transcription factor of interest. The effect on reporter gene expression of exposing such transfected cells to oligonucleotides of the invention may be used as a measure of the effectiveness of the oligonucleotides in entering the cells and inhibiting TF binding. Any suitable reporter gene may be used. The term "reporter gene," as used herein, refers to any detectable gene product; reporter gene products which are easily and inexpensively detected are preferred. It may be desirable, under certain circumstances, to use a reporter gene that encodes a product for which a highly sensitive assay is available. For example, and not by way of limitation, a highly sensitive radioimmunoassay is available for human growth hormone (HGH). Accordingly, a specific assay system, such as HeLa cells which express tat protein transfected with a transcription template that comprises the gene for HGH under the control of the HIV-1 LTR, may be used to test the efficacy of oligonucleotides inhibiting tat binding in a non-limiting embodiment of the present invention.

Oligonucleotide sequences which may represent TF binding sites may be identified by techniques including mutational analysis, footprinting studies, and gel retardation analysis as described above. Oligonucleotide sequences which have been associated with TF binding and HIV-1 transcriptional regulation, and which may be used

according to the invention, are presented in Table II. Binding site sequences which are as yet to be characterized are also provided for by the invention.

TABLE II

OLIGONUCLEOTIDE SEQUENCES USEFUL FOR INHIBITING HIV-1 TRANSCRIPTION

	REGION	REFERENCE
GGGACTTCC	Kappa enhancer	Wu et al., 1988, EMBO J. 7:2117-2130
CTCTCTGG	TAR	"
GGGCGG	SP-1	Gidoni et al., 1984, Nature 312:409-413
TGAGTCAG	AP-1	Angel et al., 1987 Cell 49:729-739
C/GTGACTC/AA	AP-1	Lee et al., 1987, Cell 49:741-752

5.3. OLIGONUCLEOTIDES OF THE INVENTION

Oligonucleotides may be synthesized using any technique used in the art. The present invention construes oligonucleotides to mean a series of nucleotides linked together, and includes nucleotides linked in a standard 5'-3' phosphodiester linkage and also molecules comprising a methylated nucleotide or nucleotide similarly modified, or any nucleoside analogue or enantiomer, or nucleosides joined by phosphorothioate linkage or molecules which comprise a variety of chemical linkages (e.g.

phosphodiester and phosphorothioate). In preferred embodiments of the invention, an oligonucleotide comprises at least one phosphorothioate linkage. Phosphorothioate modification of an oligonucleotide has been observed to enhance TF inhibition, and represents a preferred embodiment of the invention. Furthermore, as shown in example section 7, infra, phosphorothioate-linked oligonucleotides are able to reach higher concentrations in cells compared to phosphodiester-linked oligonucleotides.

In a preferred embodiment, phosphorothioate-linkages may be enzymatically introduced into double-stranded oligonucleotides of the invention. For example, and not by way of limitation, phosphorothioate linked oligonucleotides may be prepared by a reaction comprising commercially available alpha-thio-nucleotides and primers which include putative TF-binding sequences, utilizing polymerase chain reaction (PCR) technology (Saiki et al., 1985, Science 230:1350-1354).

The oligonucleotides of the invention comprise at least a portion which is double stranded, and include oligonucleotides with blunt (double-stranded) ends as well as oligonucleotides with single stranded "overhangs", in which the ends of the molecule are extensions of single-stranded nucleotide sequence beyond a double stranded nucleotide sequence beyond a double stranded region. It has been observed that oligonucleotides which comprise a single-stranded "overhang" are more effective in inhibiting TF binding, and accordingly represent preferred embodiments of the invention.

The oligonucleotides of the invention comprise one or, preferably, more than one binding site for a transcription factor. Oligonucleotides of the invention may also comprise binding sites for more than one species

of transcription factor; this may be particularly useful if two transcription factors function coordinately to control transcription of a gene.

It may be desirable for the oligonucleotides of the invention be linked to molecules which may aid the ability of the oligonucleotides to physically reach the transcriptional apparatus. Examples may include molecules with hydrophobic regions which may facilitate the penetration of the oligonucleotide through the cell membrane. Alternatively, the oligonucleotides may be linked to a nuclear localization signal (such as is utilized by SV40). Alternatively, oligonucleotides of the invention may be targeted toward a particular cell type or tissue (e.g. virus-infected cells) by an antibody specific for that cell type or tissue; oligonucleotide may be released from the antibody and then function to inhibit transcription in a particular cell type or tissue. Further, oligonucleotides may be comprised in liposomes or microcapsules, which also offers the advantage of preventing degradation of the oligonucleotides prior to cellular uptake.

5.4. UTILITY OF THE INVENTION

The present invention may be used to either increase or decrease the transcription of a gene or genes, the transcription of which is regulated by the interaction of a control element of the gene or genes and a transcription factor. According to the invention, oligonucleotides are used to inhibit the binding of the transcription factor to its control element. If the transcription factor normally acts to increase transcription, then transcription may effectively be decreased by competing oligonucleotides. If the

transcription factor normally acts to repress transcription, then transcription may be effectively increased by competing oligonucleotides.

According to one specific embodiment of the invention, the transcription factor to be inhibited is SP1. According to other specific embodiments of the invention, the transcription factor is the HIV-1 tat protein, or, alternatively, the HTLV-I tax protein, or NF-kappa B.

Transcription factors may specifically influence the expression of some genes, but not others; for example, some TFs specifically influence the transcription of viral genes, and others specifically influence the transcription of genes in certain tissues (including, but not limited to, pituitary or lymphoid cells). The specificity of TFs enables the manipulation of the transcription of genes of interest via the specific inhibition of TF binding by oligonucleotides.

For TFs which are not optimally specific, it may be desirable to direct oligonucleotides to a subpopulation of cells which utilize the TF of interest. For example, a cellular TF may induce the transcription of viral as well as cellular genes; if oligonucleotide were supplied to all cells in the body of an organism, transcription of viral and cellular genes would be inhibited in a manner potentially harmful to the organism as a whole. If, however, oligonucleotides were delivered only to virus infected cells (e.g. via antibody to a viral antigen present on the surface of infected cells, or an antibody-targeted liposome or microcapsule), then only infected cells would be affected.

The use of oligonucleotides which comprise double-stranded regions offers advantages over the use of other competitive oligonucleotides such as antisense RNA. Such advantages include increased stability, particularly in the case of oligonucleotides comprising a

phosphorothioate linkage and the fact that lower concentrations of oligonucleotide may be required to effectively compete with transcription factors (present in low concentration) as compared to the concentration required to compete with mRNA (present in higher concentrations), (See Section 6.3., <u>infra</u>).

The present invention may be utilized to control the transcription rates of any gene influenced by transcription factor binding, including cellular genes and viral genes. Clinical applications may include, but are not limited to, decreasing the imbalance of expression of globin genes in thalassemia, inhibiting the transcription of oncogenes in the treatment or prevention of malignancy inhibiting the production of growth hormone in acromegaly and inhibiting the transcription of oncogenes in the treatment or prevention of malignancy. The present invention also provides for treatment of a wide variety of viral diseases, including, but not limited to, AIDS, HTLV-I infection, and papillomavirus infection.

6. EXAMPLE: INHIBITION OF IN VITRO TRANSCRIPTION BY SPECIFIC DOUBLE-STRANDED OLIGONUCLEOTIDES

6.1. MATERIALS AND METHODS

6.1.1. PLASMID CONSTRUCTIONS

The E1B transcriptional unit was the <u>Xba</u>I-<u>Sac</u>I fragment, nucleotides 1336 to 1767 of the adenovirus-2 genome inserted into a PUC18 vector (Wu, <u>supra</u> and Gineras et al., 1982, J. Biol. Chem. 257:13475-13491). A single stranded oligonucleotide complementary to nucleotides 1730 to 1750 was used for primer extension analysis of E1B transcription. The vector and primer were gifts from M. Schmidt and A. Berk, Molecular Biology Institute, University of California, Los Angeles.

The pDUG plasmid consists of the E fragment of a BalI digest of the adenovirus-2 genome inserted into a PHC 314 vector. The E fragment contains the major late promoter of the adenovirus but no SP1 binding site (Leong et al., 1988, Mol. Cell. Biol. 8:1785-1774.

6.1.2. OLIGONUCLEOTIDE SYNTHESIS

Complementary 14 mers identical to the ones used by Kadonaga, J. T. and Tjian, R., 1986, Proc. Natl. Acad. Sci. (USA), 83:5889-5893 for purification of the SP1 transcriptional factor were chemically synthesized by D. Glick, Department of Biochemistry, University of California, Los Angeles. Additional oligonucleotides were synthesized by J. Tomich, Division of Genetics, Children's Hospital of Los Angeles, or the Microchemical Core Facility at the Norris-USC Comprehensive Cancer Center. Complementary 28 mers containing the SP1 binding site were synthesized. One set, 17/19, contained no 5-methylcytosine (5-mCyt) residues, another set, 21/25, contained a single 5-mCyt residue within the SP1 binding site. A third set, 18/20, had every cytidine residue methylated. To determine the optimal conditions for inhibition of transcription, double stranded sets of oligonucleotides containing one, two or three SP1 sites were synthesized. Some oligonucleotide sets were made with both blunt ends and 4 nucleotide complementary overhangs. To determine the effect of unrelated or low SP1 affinity sequences on inhibition of E1B transcription, oligonucleotides were synthesized corresponding to binding sites for AP2, a TATA box and a low affinity binding sequence for SP1 from SV40 (Kadonaga, J. T. and Tjian, R., 1986, Proc. Natl. Acad. Sci. (USA), 83:5889-5893). An oligonucleotide containing both an SP1 site and a TATA box separated by the same number of nucleotides as in the E1B transcriptional unit was synthesized to look for a possible interaction between

the two factors. Phosphorothioate analogues of the blunt ended oligonucleotide set containing 2 SP1 sites, SP1X2B, and a self-complementary 26 mer containing the PvuI restriction site were synthesized by hydrogen phosphonate chemistry on an Applied Biosystems DNA synthesizer and by single step sulfurization following chain assembly. All oligonucleotides were purified and annealed as described (Harrington et al., 1988, Proc. Natl. Acad. Sci. (USA), 85:2066-2070).

The sequence of the E1B transcriptional unit, 14 mers, 28 mers and primer are shown in Fig. 1. The SP1 binding site and TATA box are underlined. Transcription begins at residue +1 (Wu, *supra*). The sequences of other oligonucleotides are listed in Table III.

TABLE III

SEQUENCES OF COMPETING OLIGONUCLEOTIDES

Name	Sequence
SP1S	GATCGGGGCGGGGC CCCCGCCCCGCTAG
SPIB	GATCGGGGCGGGGC CTAGCCCCGCCCCG
SP1L	GATCGGGGCGGAGA CTAGCCCCGCCTCT
TATA	CTGCATAAATAAAAAAAA GACGTATTTATTTTTTTT
AP2	GCCTGGGGAGCCTGGGGAGC CGGACCCCTCGGACCCCTCG
SP1TATA	GGGGCGGGGCTTAAAGGGTTTTTTTTATTTAT CCCCGCCCCGGAATTTCCCAAAAAAAATAAATA
SP1X2S	GATCGGGGCGGGGCGGGGGGCGGGGC CCCCGCCCCGCCCCCCGCCCCGCTAG
SP1X2B	GATCGGGGCGGGGCGGGGGGCGGGGC CTAGCCCCGCCCCGCCCCCCGCCCCG
SP1X3S	GATCGGGGCGGGGCGGGGGGCGGGGCAAGGGGCGGGGC CCCCGCCCCGCCCCCCGCCCCGTTCCCCGCCCCGCTAG

6.1.3. IN VITRO TRANSCRIPTION

MOLT 4 and BJAB human lymphoid cell lines were grown in RPMI 1640 medium containing 10% fetal calf serum; P3X mouse plasmacytoma cells were grown in DMEM medium containing 10% horse serum and HeLa cells were grown in spinner bottles in MEM containing 5% newborn calf serum.

All media contained 50mg/liter gentamicin. Cells were harvested in log phase of growth, rapidly chilled with frozen phosphate-buffered saline (PBS), centrifuged and washed in ice cold PBS. Nuclear extracts were prepared as described, dialyzed for 4-5 hours in buffer D (Dignam et al., 1983, nucleic Acids Res. 11:1475-1489), clarified by centrifugation and frozen in liquid nitrogen. The extracts contained 3-5 mg/ml protein. They were stable for several months stored in small aliquots at -70°C.

Twenty-five μl transcription mixtures contained 0.5-1.2 μl E1B plasmid DNA or pDUG plasmid DNA in a final concentration of 26 mM HEPES pH 7.9, 48 mM KCl, 6 mM $MgCl_2$, 9.6% glycerol, 0.1 mM EDTA, 0.6 mM each of ATP, GTP CTP and UTP, 0.5 mM dithiothreitol (DTT) and 0.5 mM phenylmethylsulfonylfluoride along with varying amounts of competitor oligonucleotide. The reaction was started by addition of the extract and incubated 30-90 minutes at 30°C. It was terminated by addition of a mixture of 175 μl of 200 mM NaCl, 20 mM EDTA and 1% Na dodecyl sulfate, 20 μg purified yeast t-RNA, and extracted with the addition of 100 μl phenol and 100 μl chloroform-isoamyl alcohol (19:1) to each tube. The oligonucleotides in the aqueous phase were precipitated with 0.5 M NH_4 acetate and 3 volumes of ethanol, resuspended in 200 μl 0.3 M Na acetate pH 6, reprecipitated with 3 volumes of ethanol and dried in a vacuum centrifuge.

For analysis of the mRNA transcribed, the residue from the aqueous phase was dissolved in 10 μl of 0.25 M KCl in TE buffer containing 0.17-0.24 ng of the 20 nucleotide primer 5'-phosphorylated with [^{32}P] ATP and annealed at 65° C for 30 minutes. The solution was cooled and the primer extended by incubation for 30 minutes at 37 C in a solution containing 14 mM Tris buffer pH8, 7 mM $MgCl_2$, 3.5 mM DDT, 0.2 mM each of dATP, dGTP, dCTP and TTP, 7 μg/ml actinomycin D and 0.085 U/μl MMLV referse

transcriptase (Bethesda Research Laboratories, Gaithersburg, MD, USA) in a final volume of 35 μl. The nucleic acid was precipitated with 0.3 M Na acetate and 3 volumes of ethanol, dried, and dissolved in 10 μl buffered formamide containing bromphenol blue and xylene cyanol. They were electrophoresed at 300-400 V in a 8 M urea, 10% acrylamide, 0.3% bis-acrylamide gel. Transcription of the E1B template resulted in a 50 nucleotide band. The bands were cut from the dried gels and quantified by liquid scintillation counting.

The degree of polymerization was studied by incubation of [^{32}P]-labelled oligonucleotides under the same conditions as for _in vitro_ transcription. After precipitation with ethanol, the dried residue was dissolved in running dye and electrophorsed under non-denaturing conditions at 300-400 V in a 10% acrylamide, 0.3% bis-acrylamide gel. All bands were cut from the dried gels and quantified by liquid scintillation counting.

6.2. RESULTS

6.2.1. IN VITRO TRANSCRIPTION

This primer extension assay with E1B was developed for analysis of transcriptional factors in nuclear extracts by M. Schmidt, Molecular Biology Institute, University of California, Los Angeles (Schmidt, M. and Berk, A., unpublished observations). Nuclear extracts of lymphoid cells were used in this study since we were characterizing specific nuclear binding factors in these cells (Peterson, _supra_). Preliminary experiments showed that extracts from Molt 4, BJAB or P3X cells could transcribe the E1B template with formation of the expected 50 nucleotide band. The amount of transcript increased with extract concentration, time to 90 min. and template concentration to 21 μl/ml (11 nM). With some nuclear extracts, transcription was lower at a template

concentration of 40 μg/ml. Two μg/ml α-amanitin (Sigma, St. Louis, MO) inhibited the transcription indicating that it was dependent on RNA polymerase II. $MgCl_2$ concentrations were optimal between 6 and 7.5 mM with different extracts. Spermidine (1-4 mM) inhibited transcription and decreased the effect of α-amanitin. Addition of 4.8% polyethylene glycol 20,000 MW (Baker, Phillipsburg, NJ) or 2% polyvinyl alcohol 10,000 MW (Sigma) increased transcription with some extracts. Preincubation with template before addition of NTPs did not increase the level of transcription. In competition experiments, preincubation of the oligonucleotides with the nuclear extract prior to addition of the E1B plasmid did not increase the degree of inhibition.

6.2.2. INHIBITION OF SP1-BINDING OLIGONUCLEOTIDES

Formation of the 50 nucleotide transcript was inhibited by various double stranded oligonucleotides. The percentages of control transcription at various concentrations of competing oligomers were plotted on a semilog scale and analyzed by first and second order regression lines. A radioautograph of a typical experiment with a Molt 4 nuclear extract is shown in Fig. 2.

TABLE IV

INHIBITION OF E1B TRANSCRIPTION BY DOUBLE STRANDED OLIGONUCLEOTIDES

	Percentages of control transcription			
	20%	50%		
	μg/25μl	μg/25μl	μM	molar ratio
One SP1 site				
SP1S	0.60	0.20	0.87	83
SP1B	1.10	0.20	0.87	83
SP1L	1.58	0.64	2.80	260
17/19	0.68	0.22	0.48	45
21/25	0.80	0.17	0.37	35
18/20	0.85	0.34	0.74	70
Two SP1 sites				
SP1X2S	0.33	0.15	0.35	33
SP1X2B	0.30	0.07	0.16	16
Three SP1 sites				
SP1X3S	0.20	0.05	0.07	7
Others				
TATA	1.38	0.61	2.05	200
AP2	1.37	0.65	1.97	190
SP1TATA	1.13	0.37	0.70	67

The amount of competitor oligonucleotide required to inhibit to 20% and 50% of control was determined from regression lines using Sigmaplot statistical software. The amount required for 50% inhibition is also shown as oligonucleotide concentration and the molar ratio of oligonucleotide to template DNA.

Table IV shows the concentrations of the various oligonucleotides needed to decrease EIB transcription to 20% and 50% of the control without competitor oligonucleotides. An IC20 value gives an approximate concentration needed to achieve maximal inhibition. The IC50 is also expressed as the molar ratio of oligonucleotide to template DNA.

The IC50 was 0.87 μM for SP1S and SP1B, 14 mers with sticky and blunt ends. An oligonucleotide with a low affinity SP1 binding site, SP1L, required an IC50 of 2.80 μM. The IC50 of the unmethylated 28 mer, 17/19, was 0.48 μM. The addition of a single methylated cytosine in 21/25 did not affect transcription of E1B, while the completely methylated 28 mer, 18/20, had an IC50 of 0.74 μM. Thus, the effect of a single methylation was minimal on the ability of the synthetic oligonucleotides to compete in this transcription assay. A completely methylated oligonucleotide had a decreased ability to compete for SP1.

To determine whether an oligonucleotide with more than one SP1 site on the same side of the DNA duplex would be more effective at competing for SP1 factors, oligonucleotides with two and three SP1 sites separated by 12 nucleotides were synthesized (Fig. 3). IC50 concentrations for the sticky ended oligonucleotides with one, two or three SP1 sites were 0.87, 0.35 and 0.07 μM, respectively. IC50 concentration for the blunt ended oligonucleotide with two SP1 sites was 0.16 μM. Inhibition of transcription to 20% of control followed the same pattern.

When the concentration of E1B template was varied at a constant concentration of competing oligonucleotide, the percentage inhibition of transcription changed. Greater inhibition of transcription was seen at sub-saturating concentrations of E1B template than at saturating concentrations. This suggests that molar ratios

are important in determining the efficacy of these double stranded oligonucleotides with multiple SP1 sites. A molar ratio of only 7 was needed for 50% inhibition with the oligonucleotide containing 3 SP1 sites compared to 83 for the oligonucleotides with only one SP1 site.

To assess the specificity of the inhibition, oligonucleotides were synthesized that contained a TATA binding site or an AP2 site (Mitchell et al., 1987, Cell, 50:847-861). These oligonucleotides competed for binding factors much less efficiently. IC50 concentrations ranged from 1.97 μM to 2.05 μM, approximately threefold greater than the amount required for the oligonucleotides with high affinity SP1 sites. The competition by the AP2 oligonucleotide is consistent with the interactions reported between SP1 and AP2 with DNAase protection assays (Mitchell et al., 1987, Cell, 50:847-861).

Another oligonucleotide, SP1TATA, contained a single SP1 binding site and a TATA binding site with the same spacing as in the E1B promoter. This oligonucleotide was only slightly better inhibitor than an oligonucleotide with only an SP1 site.

Oligonucleotides with blunt ends or with four base complimentary overhangs showed differences in the shape of the inhibition curves, but similar IC50 and IC20 values. During incubation with nuclear extracts, the sticky ended oligonucleotides might form higher molecular weight structures more readily than the blunt ended ones. Such higher polymers might have greater affinity for SP1 (Kadonaga, J. T. and Tjian, R., supra). Polymerization was tested directly with end labelled oligonucleotides. The amount of higher molecular weight structures formed was less than 10% of the total, with both sticky and blunt oligonucleotides after 90 minutes incubation with MOLT 4 nuclear extracts. During this incubation with blunt and sticky ended oligonucleotides were degraded to 23% and to

55% of their original amount, respectively. To show the specificity of inhibition, we tested the effect of unrelated oligonucleotides and the upper and lower strand of the 14 mer SP1S oligonucleotide. Transcription was 140% of control with 0.4 μg/25 μl and 91% of control when 1.0 μg/25 μl of a double stranded PvuI linker (N.E. Biolabs), 5'-TCGCGATCGCGA-3', was added. Addition of 0.5 μg/25 μl or 1.0 μg/25 μl of the lower strand 14 mer (Fig. 1) caused a decrease to 98% and 78% of the control, respectively. In contrast, 0.5 μg/25 μl or 1.0 μg/25 μl of the upper strand of the 14 mer caused a decrease to 71% and 35% of the control, respectively. Thus, the presence of a double stranded oligomer without a GC box did not compete for SP1, while the single stranded oligonucleotide containing a GC box did compete for SP1 binding at high concentrations. This competition by the guanine rich strand of SP1 is consistent with a report that methylation protection by SP1 occurs only on the guanine rich strand (Gidoni et al., 1984, Nature, 312:409-413).

We also tested oligonucleotides that compete for E1B transcription in assays with the E fragment of a BalI digest of the adenovirus 2 genome. This sequence contains the major late promoter of the adenovirus and no SP1 site (Leong, 1988, supra). Transcription with Molt 4 and HeLa nuclear extracts was inhibited only 20% with concentrations of competing oligonucleotide up to 1.5 μg/μl. Transcription was actually enhanced with low concentrations of the competing oligonucleotide.

We synthesized and annealed a set of phosphorothioate linked oligonucleotides with the same sequences as SP1X2B, the blunt ended set with 2 LSP1 sites. Transcription was only 5% of control values with concentrations of 0.04 μg/25 μl (0.09 μM) or greater of the phosphorothioate derivative while SP1x2B had an IC20 of 0.30 μg/25 μl. Thus, the double stranded phosphorothioate

was a more effective inhibitor than SP1X2B. A control 26 mer phosphorothioate containing a PvuI site did not inhibit EIB transcription at concentrations of 1 μg/25 μl.

6.3. DISCUSSION

In vitro assays have been used to define the factors necessary for transcription of specific genes by RNA polymerases II and III (Gidoni et al, 1984, *supra*; Chodoch et al., 1986, Mol. Cell. Biol., 6:4723-4733; Hawley, D. K. and Roeder, R. G., 1985, J. Biol. Chem. 260:8163-8172 and Bieker et al., 1985, Cell 40:119-127). These experiments have identified the basic transcriptional factors needed for each polymerase as well as nuclear binding proteins that can increase or decrease the rate of initiation. If the factors for a particular gene are known, then it may be possible to inhibit or increase transcription by competing for the nuclear factors (Mulvihill, E. R. and Chambon, P., 1983, Nature, 301:680-686). Such an approach applied in an *in vivo* system may lead to a potential new therapy.

The studies reported here indicate that addition of short double-stranded oligomers containing the binding site for one of these factors, SP1, can inhibit *in vitro* transcription of E1B. Mutants lacking the SP1 site transcribe E1B at only 13 to 20% of basal levels, *in vivo* (Wu, 1987, *supra*). Inhibition of *in vitro* transcription to this level was achieved with concentrations less than 1 μM of the 14 and 28 mers containing 1 SP1 site. A single stranded oligonucleotide containing the GGGCGG sequence required higher concentrations to compete at the same levels as double stranded oligonucleotides.

A series of experiments were initiated to define the effects of the number of binding sites and sequence on the ability of double stranded oligonucleotides to inhibit transcription. Oligonucleotides with two SP1 sites inhibited transcription 50% with a molar ratio of

oligonucleotide to template that was approximately 3 times lower than an oligonucleotide with one SP1 site. The oligonucleotide with three SP1 sites inhibited 50% with a molar ratio that was approximately 11 times lower than the one with a single SP1 site. In contrast, an oligonucleotide set containing a SP1 site and TATA box was no more effective than one SP1 site. This data suggests that there are cooperative interactions between SP1 factors but not between SP1 and TATA.

Oligonucleotides with sticky ends were generally more effective in inhibiting transcription than blunt ends. This was not due to polymerization but may be due to better resistance to degradation.

Methylation of DNA has been proposed as an important component in the control of expression of certain eukaryotic genes (Yisraeli, J. and Azyl, M., 1984, DNA Methylation, 353-378). Several groups have shown that methylation of cytosine residues in GC boxes does not alter binding to SP1 (Harrington, 1988, supra, Hoeveler, A. and Doerfler, W., 1987, DNA 6:449-460; and Holler et al., 1989, Genes and Development 21:1127-1135). The present experiments assay confirm the lack of a direct effect of methylation of single residues on transcription regulated SP1 binding to the GC box. Multiple methylated sites had a slight effect on the ability of the oligonucleotide to bind to SP1. This may be due to greater steric hindrance.

Phosphorothioate oligonucleotides are more resistant to degradation by nucleases but this chemistry introduces an asymmetric center at each internucleotide linkage (Zon, G., 1988, Pharmaceutical Res., 5:539-549). In our assay, a phosphorothioate linked oligonucleotide was a better inhibitor of E1B transcription than its corresponding normal oligonucleotide. Thus, the

steroisomers do not appear to prevent SP1 binding. The improved effect may be due to resistance to nucleases present in cell extracts.

The use of double stranded oligonucleotides to control gene expression may present several advantages. Double stranded oligonucleotides are designed to bind nuclear binding factors rather than mRNA molecules. In most cases, there are few copies of transcriptional elements for a specific gene and few molecules of nuclear molecules of nuclear binding factors relative to the number of RNA transcripts. This advantage is illustrated by our data showing that the concentrations of double stranded oligonucleotides that inhibit transcription are 10 to 100 times lower than the reported concentrations of antisense oligonucleotides needed to block expression of various genes _in vitro_ (Stein, 1988, _supra_). Our experiments have defined some specific parameters for double stranded oligonucleotides to achieve optimal inhibition of _in vitro_ transcription of E1B. Similar experiments with more complex promoters may define interactions of their nuclear binding factors.

7. EXAMPLE: UPTAKE OF DOUBLE-STRANDED PHOSPHOROTHIOATE OLIGONUCLEOTIDES

It has been shown that double-stranded oligonucleotides with phosphorothioate linkages were taken up much more efficiently by MOLT 4 human leukemia cells in culture. As shown in Figure 4, the uptake of radiolabelled phosphodiester-linked SP1X2B oligonucleotides was significantly less than that of radiolabelled phosphorothioate-linked oligonucleotide SP1X2m which also has two SP1 sites. This difference in uptake may in fact reflect a difference in the stability of the oligonucleotides once inside the cell. This is supported by the observation that the uptake curves for both

oligonucleotides in Figure 4 were initially roughly parallel. Subsequently, however, the curve for phosphodiester-linked oligonucleotide uptake formed a plateau, whereas the curve for phosphorothioate-linked oligonucleotide continued to rise. This suggests that phosphorothiate-linked oligonucleotides had accumulated, but phosphodiester-linked oligonucleotides were degraded.

The present invention is not to be limited in scope by the specific embodiments described herein. Indeed, various modifications of the invention in addition to those described herein will become apparent to those skilled in the art from the foregoing description and accompanying figures. Such modifications are intended to fall within the scope of the appended claims.

Various publications are cited herein, the disclosures of which are incorporated by reference in their entireties.

WHAT IS CLAIMED IS:

1. A method for controlling the expression of a gene comprising inhibiting the binding of a transcription factor to a transcriptional control element of the gene by competitively binding the transcription factor to a double-stranded oligonucleotide that comprises at least one phosphorothioate linkage.

2. The method of claim 1 which increases transcription of the gene.

3. The method of claim 1 which decreases the transcription of the gene.

4. The method of claim 3 in which the gene is a cellular gene.

5. The method of claim 3 in which the gene is a viral gene.

6. The method of claim 5 in which the viral gene is an adenovirus gene.

7. The method of claim 5 in which the viral gene is a papillomavirus gene.

8. The method of claim 5 in which the viral gene is a retrovirus gene.

9. The method of claim 8 in which the retrovirus is a causative agent of acquired immunodeficiency syndrome.

10. The method of claim 9 in which the retrovirus is human immunodeficiency virus 1.

11. The method of claim 10 in which the transcription factor is a product of the tat gene.

12. The method of claim 10 in which the transcription factor binds to the viral enhancer element.

13. The method of claim 10 in which the transcription factor binds to the negative regulatory region of the viral genome.

14. The method of claim 10 in which the transcription factor binds to an SP-1 site in the viral genome.

15. The method of claim 10 in which the transcription factor binds to the TAR region of the viral genome.

16. The method according to claim 3 in which the oligonucleotide comprises the sequence GGGCGG or at least a 4 bp subsequence thereof.

17. The method of claim 3 in which the oligonucleotide comprises the sequence GGACTTCC or at least a 4 bp subsequence thereof.

18. The method according to claim 3 in which the oligonucleotide comprises the sequence CTCTCTGG or at least a 4 bp subsequence thereof.

19. The method according to claim 9 in which the oligonucleotide comprises the sequence GGGCGG or at least a 4 bp subsequence thereof.

20. The method of claim 9 in which the oligonucleotide comprises the sequence GGACTTCC or at least a 4 bp subsequence thereof.

21. The method according to claim 9 in which the oligonucleotide comprises the sequence CTCTCTGG or at least a 4 bp subsequence thereof.

22. A composition for inhibiting the expression of a gene comprising a double-stranded oligonucleotide which comprises the sequence GGACTTCC or at least a 4 bp subsequence thereof and which comprises at least one phosphorothioate linkage.

23. A composition for inhibiting the expression of a gene comprising a double-stranded oligonucleotide which comprises the sequence CTCTCTGG or at least a 4 bp subsequence thereof and which comprises at least one phosphorothioate linkage.

24. A composition for inhibiting the expression of a gene comprising a double-stranded oligonucleotide which comprises the sequence GGGCGG or at least a 4 bp subsequence thereof and which comprises at least one phosphorothioate linkage.

FIG. 1

E1b -65 TO +50

```
   -60       -50       -40       -30       -20       -10
    .         .         .         .         .         .
TGCATGGCGTGTTAAATGGGGCGGGGCTTAAAGGGTATATAATGCGCCGTGGGCTAATCTTGGTT

                 GATCGGGGCGGGGC    14-mer
                     CCCCGCCCCGCTAG

       ACGTTGCAGCCGGGGCGGGGCTTCTGCA    28-mer
           ACGTCGGCCCCGCCCCGAAGACGTTGCA

1        10        20        30        40        50
.         .         .         .         .         .
ACATCTGACCTCATGGAGGCTTGGGAGTGTTTGGAAGATTTTTCTGCTGTGC
                                CCTTCTAAAAAGACGACACG
```

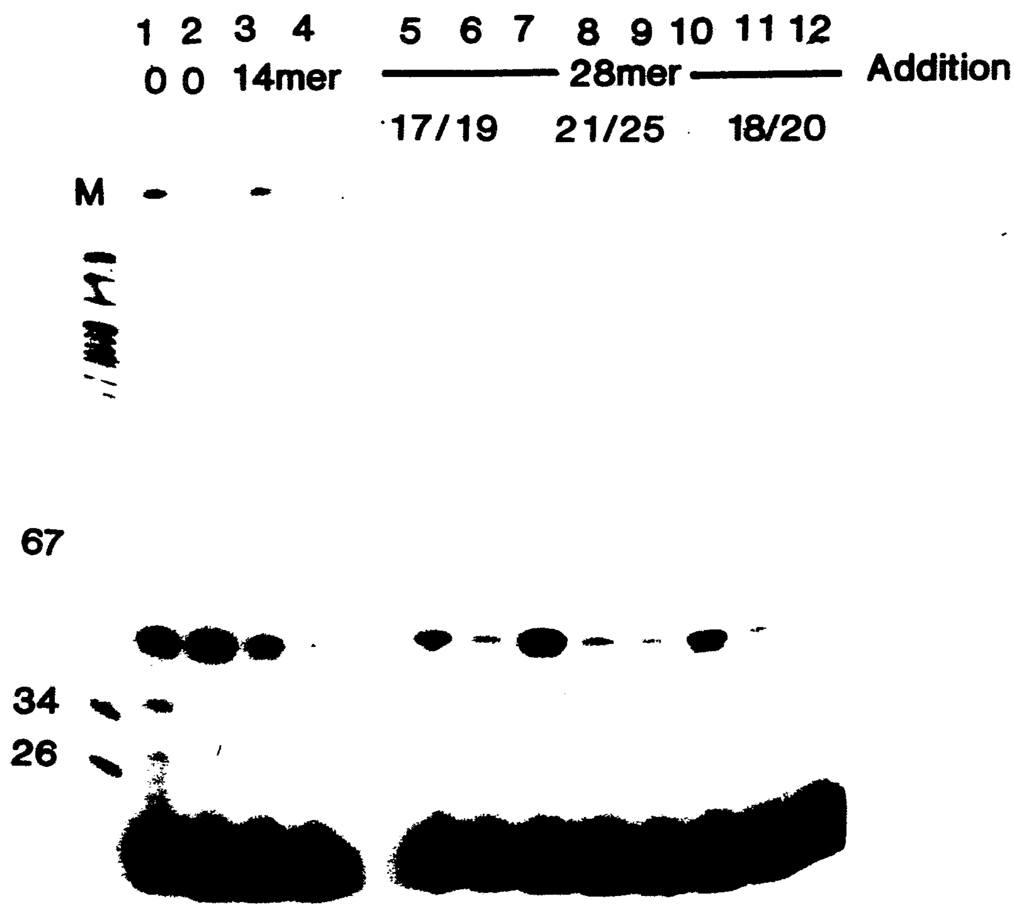
1 2 3 4 5 6 7 8 9 10 11 12
0 0 14mer 28mer Addition
17/19 21/25 18/20
M
67
34
26

FIG. 2

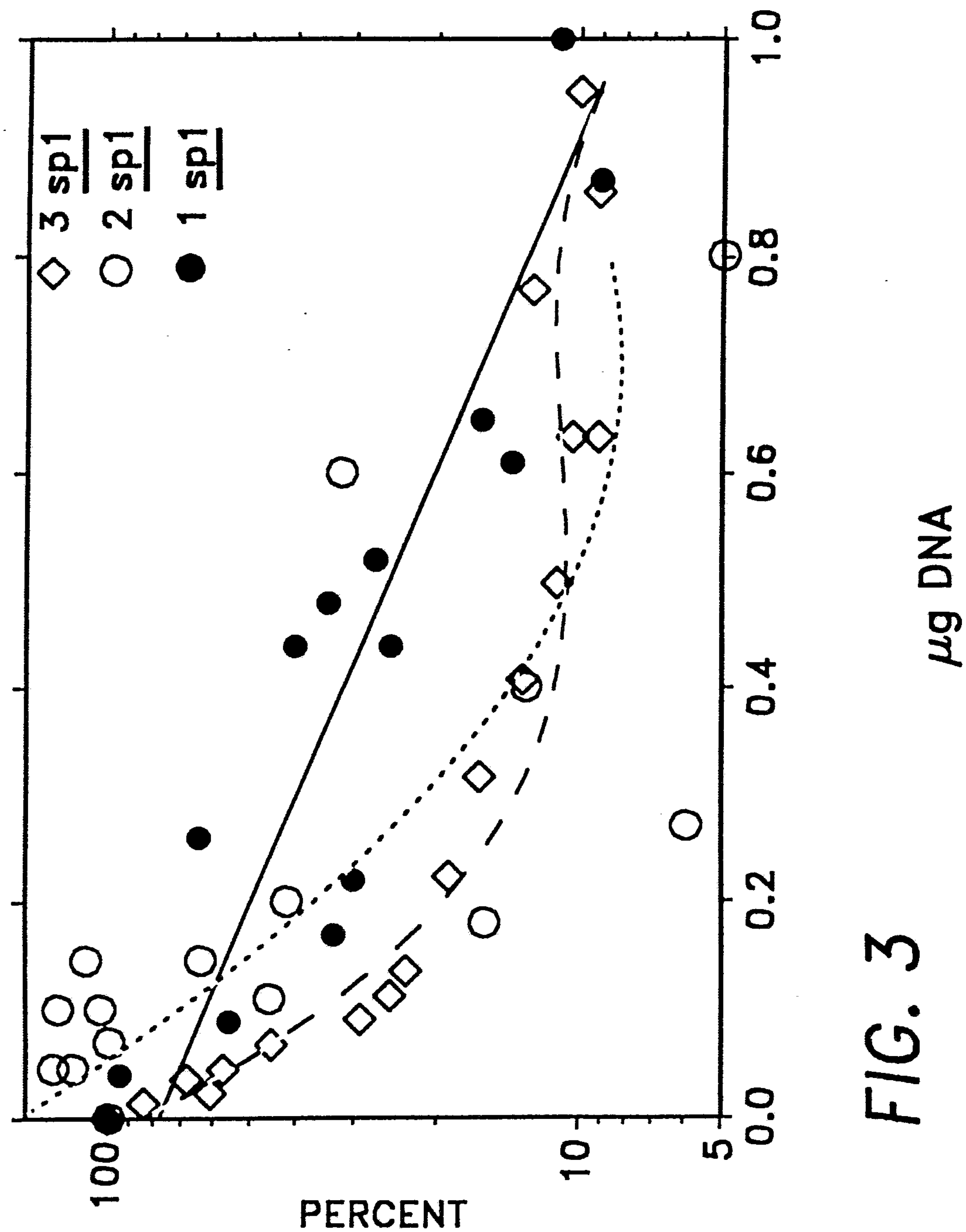

FIG. 3

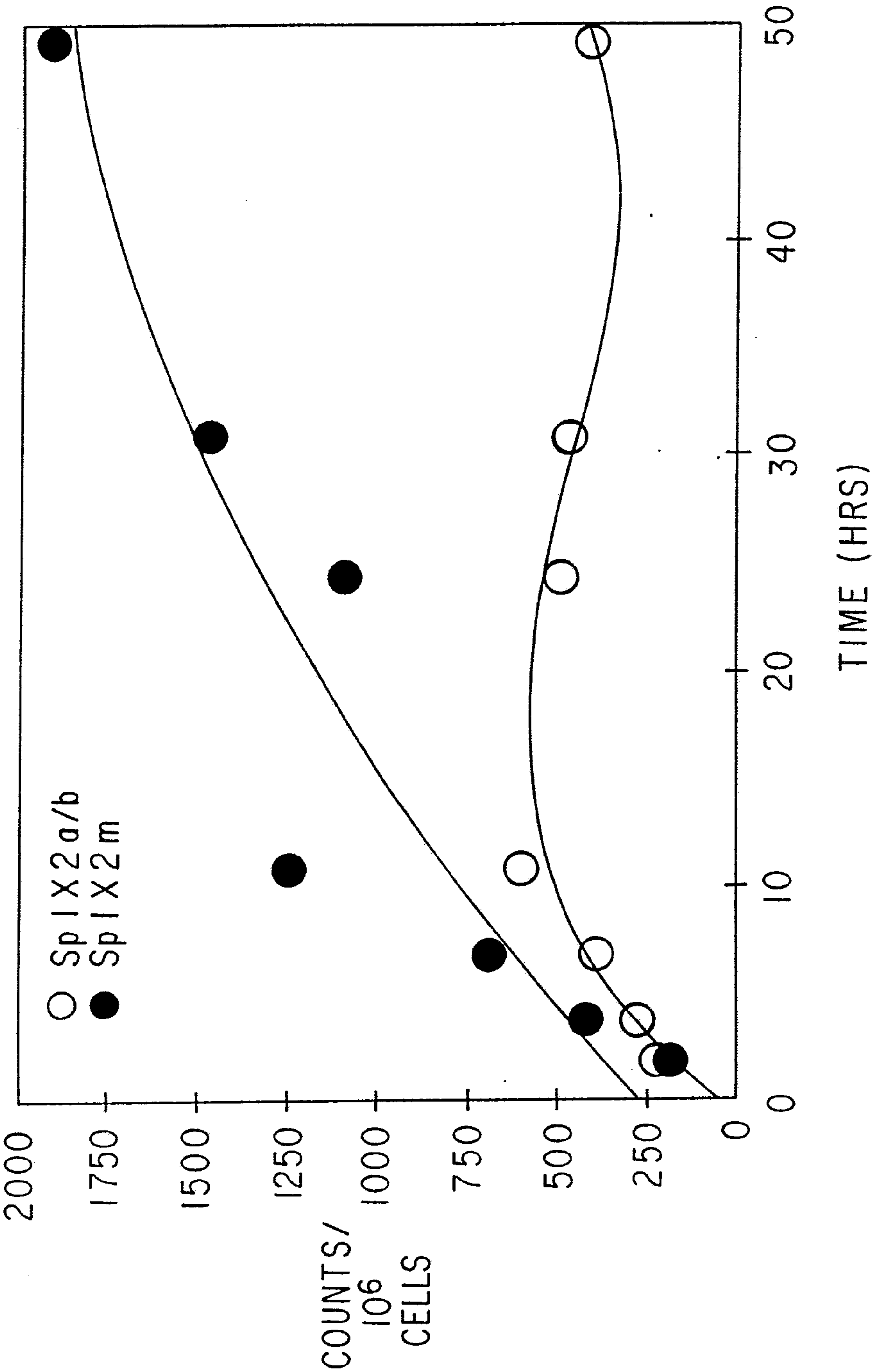
COUNTS/
10^6
CELLS
TIME (HRS)
0
250
500
750
1000
1250
1500
1750
2000
0
10
20
30
40
50
Sp I X 2 a/b
Sp I X 2 m

FIG. 4

INTERNATIONAL SEARCH REPORT

International Application No. PCT/US91/00635

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [6]

According to International Patent Classification (IPC) or to both National Classification and IPC

I.P.C.(5): C12Q 1/68; C07H 15/12; C12N 15/00

U.S.Cl. 435/6; 536/27; 935/77,78

II. FIELDS SEARCHED

Minimum Documentation Searched [7]

Classification System	Classification Symbols
U.S.Cl.	435/6; 536/27; 935/77,78

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched [8]

GenBank, EMBL, STN

III. DOCUMENTS CONSIDERED TO BE RELEVANT [9]

Category *	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No. [13]
X Y	Nucleic Acids Research, Vol. 17, No. 21, issued 1989, Latchman et al, The Different Competitive Abilities of viral TAAGARAT Elements and cellular octamer Motifs, Mediate the induction of viral Immediate-Early Genes and the Repression of the Histone H2B Gene in Herps Simplex Virus infected cells" pages 8533-8542, see entiredocument.	1-5,8 6,7,9-21
X Y	Nature, Vol. 312, issued 29 November 1984, Gidoi et al, "Multiple specific contacts Between a Mammalian Transcription Factor and its cognate Promoters" pages 409-413, see entire document.	24 6,7,9-21

* Special categories of cited documents: [10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search

07 March 1991

Date of Mailing of this International Search Report

26 APR 1991

International Searching Authority

ISA/US

Signature of Authorized Officer

Samuel Rassie for

Mindy B. Fleisher

Form PCT/ISA/210 (second sheet) (Rev.11-87)

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
X Y	The EMBO Journal, Vol. 7, No. 7, issued 1988 Wu et al, "Purification of the Human immunodeficiency virus type 1 enhancer and TAR Binding Proteins EBP-1 and UBP-1" pages 2117-2129, see entire document.	22,23 6,7,9-21
Y	Cell, vol. 50, issued 03 July 1987, Lambert etal, "A Transcriptional Repressor Encoded by BPV-1 shares a common carboxy terminal Domain with the E2 transactivator" pages 69-78, see entire document.	6,7,9-21
Y	The EMBO Journal, Vol. 8, No. 3, issued 1989, Garcia etal, "Human immunodeficiency virus Type 1 LTR TATA and TAR Region Sequences Required for Transcriptional Regulation" Pages 765-778, see entire document.	6,7,9-21
Y	Science, Vol. 229, issued 05 July 1985, Arya etal, "Trans-Activator Gene of Human T-Lymphotrocpic Virus Type III (HTLV-III)" pages 69-73, see entire document.	6,7,9-21